Lentiviral shRNA screen of multidrug resistant associated genes identifies PRP-4 as a new regulator of chemoresistance in human ovarian cancer

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ABSTRACT

Experimental evidence implicates a wide range of mechanisms that may contribute to the drug resistant phenotype in ovarian cancer. The major goal of this study is to determine the mechanism underlying the relationship between overexpression of drug resistance associated genes and multidrug resistance in ovarian cancer. Using lentiviral shRNAs targeting 120 genes identified from transcriptionally profiling drug resistant cancer cell lines, individual knockdown experiments were performed in the presence of sublethal doses of paclitaxel. Specific knockdown associated cellular toxicity identified ABCB1 (MDR1), survivin and PRP-4 (Pre-mRNA Processing factor-4) as genes directly linked to paclitaxel resistance in the paclitaxel resistant cell line SKOV-3$_{TR}$. Both ABCB1 and survivin have been previously reported to play a role in multidrug resistance and chemotherapy drug induced apoptosis, while the effects of PRP-4 expression with drug sensitivity is currently unrecognized. PRP-4 belongs to the Ser/Thr protein kinase family, plays a role in pre-mRNA splicing, and interacts with CLK1. Northern analysis demonstrated that PRP-4 is overexpressed in several paclitaxel-resistant cell lines and also confirmed PRP-4 expression could be significantly knocked down by PRP-4 lentiviral shRNA. Both clonegenic and MTT assays demonstrated that knock down of PRP-4 could reverse paclitaxel resistance 5-10 fold in the paclitaxel resistant cell line SKOV-3$_{TR}$. Finally, overexpression of PRP-4 in drug sensitive cells could induce a modest level of drug resistance to paclitaxel.

MATERIALS AND METHODS

Cell Culture. The human ovarian cancer cell line SKOV-3 used in this study was obtained from the American Type Culture Collection (Rockville, MD). The paclitaxel resistant SKOV-3$_{TR}$ was established as previously reported and cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin (all obtained from Invitrogen, Carlsbad, CA). Resistant sub clones were continuously cultured in paclitaxel. Paclitaxel was obtained through unused residual clinical material at the Massachusetts General Hospital.

Drug Resistance Associated Genes Lentiviral shRNA Library. The genes were chosen based on their relevance to multidrug resistance in human ovarian cancer from our previous studies. Based on 291 gene transcripts that demonstrated over-expression in at least two resistant cell lines, we constructed a lentiviral shRNA library composed of 132 genes at Sigma-Aldrich Research Biotechnology (St. Louis, MO). Details of the library-production methods can be found at the Sigma web site (http://www.sigmaaldrich.com/Area_of_Interest/Life_Science/Functional_Genomics_and_RNAi/shRNA). In particular, these genes were pre-selected because they also included full-length open reading frames (ORF). These ORF will be useful for confirmatory functional studies in which full-length transcripts would be transfected back into sensitive cell lines.

Lentiviral Infection. Infection conditions in SKOV-3$_{TR}$ were optimized with MISSON® TurboGFP™ Control Transduction particles (SHC003V) and Non-target shRNA Control Transduction particles (SHC002V) (Sigma) in 96-well plates for optimal growth conditions, viral dosage, puromycin selection concentration and assay times prior to screening. On day 1, SKOV-3$_{TR}$ cells were seeded at 1.6x10^4 cells/well in triplicate, on 96-well plates and labeled with plate A, B or C, and incubated for 24 hr at 37°C (Fig. 1). On day 2, 8 µg/ml of hexadimethrine bromide were added to each well, and infected using 8 µl of lentiviral particles encoding shRNA against different drug resistance associated genes to appropriate wells, and incubated for 24 hr at 37°C. On day 3, plate A was kept as the control without the addition of puromycin or paclitaxel, the shRNA viral particle-containing medium was removed and replaced it with fresh medium. For plate B, viral particle-containing medium was removed and replaced it with fresh medium containing 5 µg/ml of puromycin for selection of transduced cells. For plate C, viral particle-containing medium removed and replaced it with fresh medium containing 5 µg/ml of puromycin and 0.1 µM of paclitaxel. From day 4 to day 10, fresh medium was replaced with necessary as above described and examined if there were any cytotoxicity in any wells under the microscope. On Day 10, the number of viable cells was determined via CellTiter 96®AQ$_{ueous}$ One Solution Cell Cytotoxicity Assay (Promega, Madison, WI)

Determination of the Results and Analysis. The general format was designed to include evaluation of each target gene through shRNA lentiviral- mediated gene knockdown. The experiments were conducted in two control plates and one experimental plate to permit plate-to-plate comparisons (Fig. 1). The control plates (plate A and plate B) were used to evaluate transduction efficiency, and to exclude target genes that were lethal during knockdown in the absence of chemotherapy. The latter control (plate B) was necessary because the goal of this study was to identify genes that are important in acquired drug resistance and not cell survival. Therefore, one plate (plate A) was given only lentivirus to confirm that the shRNA is not lethal in the absence of puromycin and paclitaxel (a positive result is cell survival); A second plate (plate B) was exposed to puromycin and lentivirus to confirm efficiency of infection (once again a positive result is cell survival). The third plate (plate C) was given both lentiviral shRNA and 0.1 µM paclitaxel. This is typically a sublethal dose of paclitaxel for the SKOV-3$_{TR}$ cell line and a positive result would be cell death at 4-10 days. shRNA targeted genes that are associated with cells surviving in plate A and B and dying in plate C was identified as "hits" and selected for further study. Each of the 132 genes is represented by three to five different shRNA lentiviral particle constructs targeting different sites of the genes. In addition, since these shRNA are complimentary to different regions of the mRNA, it reduces the risk of "off target effects". To further minimize the possibility of "off target" hits, we only focused on genes identified as functional drug resistance genes by two or more shRNA targeting the same gene. All experiments used cells infected with a non-target vector control virus (Sigma) as a negative control for expression changes. Once hits were identified, we validated that the targeted genes indeed have been knocked down by RT-PCR and Northern analysis.

RNA Extraction: RNA was collected from SKOV-3, SKOV-3$_{TR}$. Total RNA was isolated using TRIzol® Reagent (Invitrogen) according to the manufacturer's instructions. Additionally, RNA quality was determined *via* ethidium bromide staining followed by agarose/formaldehyde gel electrophoresis.

RT-PCR. For the PRP-4 gene, the results from the shRNA screening were verified using RT-PCR and Northern blot analysis. RT-PCR was performed using the sense and antisense primers to human PRP-4 sense primer 5'-ATAAGAATGCGGCCGCGGAAGTTCAAGATGGCCGCCG-3' to introduce a Not I site as underlined, and antisense primer: 5'-GGTGGATCCCACACACTCAAACCCTTGGAG-3' (GenBank # NM_003913) to introduce a BamH I site as underlined. The introduced restriction enzyme sites were designed for following confirmatory functional studies. TRIzol extracted total RNA was DNAase-treated to remove contaminating genomic DNA according to the manufacturer's protocol (Invitrogen). RT-PCRs were performed using the Titan One Tube RT-PCR systems (Roche, Indianapolis, IN) following the manufacturer's protocol.

Northern Analysis. PRP-4 RT-PCR products were cloned into the pCR®2.1 vector using a TA Cloning® kit (Invitrogen) and the cloned fragment was confirmed by sequencing. The cDNA inserts were cut using Not I and BamH I (Promega) and purified using the QIAEXII Gel Extraction Kit (Qiagen Inc, Chatsworth, CA). PRP-4 probes were labeled with ^{32}P-dCTP using the Megaprime DNA Labeling System. (Amersham Pharmacia Biotech, Piscataway, NJ). Total RNA was extracted using the TRIzol reagents as described above. RNA was separated by electrophoresis in 1.2% agarose/formaldehyde gels (approximately 5µg total RNA per lane), transferred to Hybond N-plus nylon membranes (Amersham Biosciences) and UV cross-linked. A one hour prehybridization step was performed in Rapid-hyb buffer (Amersham Biosciences), followed by a 2 hour hybridization of the ^{32}P-labeled PRP-4 probe in the same buffer. The blots were washed twice at room temperature with 2XSSC-0.1%SDS for 15 min and twice at 65 □C with 0.2XSSC-0.1% SDS for 15 min. Blots were exposed to autoradiography X-ray films with an intensifying screen. Finally, to confirm the amounts of RNA loaded in each lane, blots were hybridized with an β-actin probe. Relative quantification was performed using densitometry.

Cytotoxicity Assay. Paclitaxel cytotoxicity was assessed in vitro using the MTT assay as previously described. Briefly, 2×10^3 cells per well were plated in 96-well plates in culture medium (RPMI 1640 supplemented with 10% fetal bovine serum and penicillin/streptomycin) containing increasing concentrations of paclitaxel. After 7 days of culture, 10 µl MTT (5 mg/ml in PBS, obtained from Sigma) were added to each well and the plates were incubated for 4 h. The resulting formazan product was dissolved with acid-isopropanol and the absorbance at a wavelength of 490 nm (A_{490}) was read on a SPECTRAmax® Microplate Spectrophotometer (Molecular Devices, Sunnyvale, CA). The absorbance values were normalized by assigning the value of the control line in the medium without drug to 1.0 and the value of the no cell control to 0. Experiments were performed in triplicate. Dose response curves were fitted with use of GraphPad PRISM® 4 software (GraphPad Software, San Diego, CA).

PIRES$_{PRP-4}$ Expression Vector Construction. Clonetech's (Palo Atlo, CA) mammalian expression vector pIRESneo was used for functional expression study. The expression cassette of pIRESneo contains the human cytomegalovirus major immediate early promoter/enhancer (pCMV) followed by a multiple cloning site (MCS) and a synthetic intron known to enhance the stability of the mRNA. A 3094 base pair cDNA fragment containing the full ORF of human PRP-4 was amplified by RT-PCR from the RNA of SKOV-3$_{TR}$, a paclitaxel resistant cell line that highly overexpresses PRP-4. The resulting PRP-4 RT-PCR product was cloned to pCR®2.1 vector using Invitrogen's Original TA Cloning Kit. After sequence confirmation, PRP-4 was cut from the pCR®2.1 vector, purified, subcloned to the MCS of expression vector pIRESneo, and subsequently sequenced to confirm the correct ORF. Expression of PRP-4 cDNA was under the control of the pCMV.

Transfection and Production of Stable Cell Lines. Transfections were performed using LipofectAmine Plus reagents (Invitrogen) as follows: approximately 5x10^5 SKOV-3 cells were plated into 90 millimeter tissue culture dishes and cultured overnight. Prior to transfection, the growth medium was replaced with serum free RPMI 1640 and cultured for three hours. LipofectAmine reagent containing 5 µg of pIRES$_{neo}$, pIRES$_{PRP-4}$ was combined with Plus reagent and applied to the cells. After culture for four hours, the media was replaced with RPMI 1640 containing 10% fetal bovine serum. G418 sulfate (Invitrogen) selection (300µg/ml) was started at 24 hours post transfection. The selection medium was changed every 2 days.

Western Blotting. The human PRP-4 antibody was generously provided by Dr. Regis Giet (University Rennes, France). The Pgp antibody C219 was purchased from Signet (Dedham, MA). The mouse monoclonal antibody tohuman β-actin was purchased from Sigma-Aldrich. PRP-4 and Pgp proteins were analyzed in total cell lysates. Total cell lysates were prepared, and Western blot analysis was performed as previously described. Briefly, the cells were lysed in 1X RIPA lysis buffer (Upstate Biotechnology, Charlottesville, VA) and protein concentration was determined by the DC Protein Assay (Bio-Rad). Twenty-five micrograms of total protein were resolved on NuPage™ 4-12% Bis-Tris Gels (Invitrogen) and immunoblotted with specific antibodies. Primary antibodies were incubated in TBS (pH 7.4) with 0.1% Tween-20 with gentle agitation overnight at 4°C. Horseradish peroxidase (HRP)-conjugated secondary antibodies (Bio-Rad) were incubated in TBS (pH 7.4) with 5% nonfat milk (Bio-Rad) and 0.1% Tween-20, at a 1/2000 dilution for one hour at room temperature with gentle agitation. Positive immunoreactions were detected by using SuperSingal® West Pico Chemiluminescent Substrate (Pierce Biotechnology, Inc. Rockford, IL).

INTRODUCTION

Standard chemotherapy for newly diagnosed and recurrent ovarian cancer includes a combination of paclitaxel and carboplatin. Although objective responses and survival benefits are seen, the efficacy of both of these agents is limited by the eventual development of multidrug resistance (MDR). There is little understanding of how ovarian tumors, as well as other tumors, develop drug resistance. Experimental evidence from several laboratories implicates a wide range of mechanisms that contribute to the drug resistant phenotype and reversing drug resistance has been a high priority goal of clinical and investigational oncology.

Gene expression profile analysis is an efficient technology that allows screening for correlations between expression of specific genes and acquisition of multidrug resistance. In an attempt to identify novel genes that are differentially expressed between paclitaxel-resistant and paclitaxel sensitive cells, the paclitaxel-sensitive ovarian cancer cell lines, SKOV-3 and OVCAR8, and the paclitaxel sensitive breast cancer cell line, MCF-7, were exposed to incrementally increasing concentrations of paclitaxel. This procedure resulted in the establishment of three paclitaxel resistant daughter cell lines, SKOV-3$_{TR}$, OVCAR8$_{TR}$ and MCF-7$_{TR}$, respectively. Gene expression in these three paclitaxel-resistant cell lines and their corresponding sensitive parental lines (SKOV-3 *vs* SKOV-3$_{TR}$; OVCAR8 *vs* OVCAR8$_{TR}$; MCF-7 *vs* MCF-7$_{TR}$) was characterized using Affymetrix microarray technology. A large number of transcripts were identified as differentially expressed between sensitive and resistant cell lines. 790 (SKOV-3$_{TR}$), 689 (OVCAR8$_{TR}$) and 964 (MCF-7$_{TR}$) gene transcripts demonstrated more than a two-fold overexpression in the paclitaxel resistant lines relative to their expression in the sensitive parental lines. Although SKOV-3$_{TR}$, OVCAR8$_{TR}$ and MCF-7$_{TR}$ all demonstrate a paclitaxel resistant phenotype, the transcripts identified with altered expression in each cell line pair were largely non-overlapping and encoded proteins with a wide variety of biochemical functions. Recently, we have identified a collection of approximately 132 genes that are overexpressed in multiple paclitaxel- resistant cell lines as compared to their paclitaxel- sensitive parental lines. However, establishing a strong correlation between drug sensitivity and particular gene expression has proved to be challenging.

The ability to use RNA interference (RNAi) as a tool for functional gene silencing in tumor cells has enabled us to perform loss- of- gene function studies in tissue culture systems. But while siRNA has been shown to be effective for short-term gene inhibition in mammalian cell lines, there is a clear problem in its use for stable transcript knockdown. Recently, short hairpin RNA (shRNAs) libraries in lentiviral vectors have been described and used in stable cell lines and in transgenic mice. Our aim was to identify genes essential for drug resistance by screening for shRNAs that selectively reverse drug resistance in cancer cell lines. This type of screen holds promise for the discovery of novel targets to reverse drug resistance in cancer therapy and genetically validated combination therapies.

In present study, we utilized the above-mentioned collection of 132-lentiviral shRNA library targeting the expression of drug resistance associated proteins to address whether loss of these proteins impact paclitaxel sensitivity *in vitro*. This pre-selected shRNA library was drawn from our previous cDNA array studies; the screen has been carried out in the well-characterized ovarian cancer paclitaxel- resistant cell line SKOV-3$_{TR}$. We show that inhibition of several target genes could sensitize SKOV-3$_{TR}$ to paclitaxel. Specifically, we describe the overexpression of human pre-messenger RNA processing (PRP-4) kinase in ovarian cancer multidrug resistant cells and specific PRP-4 knockdown associated cellular paclitaxel sensitivities. Furthermore, we have identified a set of drug resistance associated genes that, when silenced, cause cell sensitivity to paclitaxel, several of which (such as ABCB 1/MDR1, MDM2, and survivin) have been previously shown to be involved in multidrug resistance. This observation suggests that overexpression of PRP-4 is a new mechanism for the acquisition of paclitaxel resistance and therefore a potential novel target for the development of therapies treating drug resistance in ovarian cancer.

Flow chart of the functional lentiviral shRNA screen

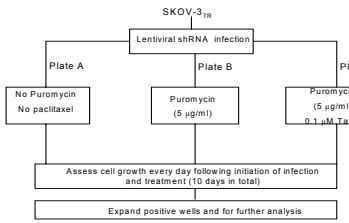

Figure 1. Flow chart of the functional lentiviral shRNA screen measuring the effect of drug resistant gene knockdown on paclitaxel sensitivity. The drug resistance associated 132-lentiviral shRNA library was established. For the screen, 1.6x10^4 SKOV-3$_{TR}$ cells per well in 96-well plates were infected with lentiviral particles encoding shRNA against different drug resistance associated genes. The infected cells were subsequently selected with puromycin following a protocol provided by the manufacturer. The number of viable cells was analyzed using the CellTiter 96®AQ$_{ueous}$ One Solution Cell Cytotoxicity Assay and visualized under the microscope.

Table 1. The list of positive hits from the lentiviral shRNA screen in SKOV-3$_{TR}$

Gene name	Symbol	Functions	Positive hits
Pre-mRNA Processing factor 4	PRP-4	pre-mRNA splicing, Interacts with Clk1 C-terminus	3 out 5
Survivin	EPR-1	Inhibit apoptosis, regulate mitosis	2 out 4
Multidrug resistance 1	MDR1	efflux pump responsible for decreased drug accumulation	2 out 5
Nuclear matrix protein p84	THOC1	protein transcription and RNA export	2 out 5
Oncoprotein mdm2	mdm2	Inhibit cell cycle arrest and apoptosis	2 out 4
Krev interaction trapped 1	Krit1	unknown	2 out 5

Effect of PRP-4 knockdown on paclitaxel sensitivity in SKOV-3$_{TR}$ cell

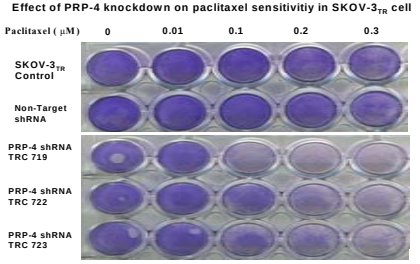

Figure 2. Effect of PRP-4 knockdown on paclitaxel sensitivity in SKOV-3$_{TR}$ cells. Clonogenic assay transduction of PRP-4 lentiviral shRNA vector confers paclitaxel sensitivity to SKOV-3$_{TR}$ cells. After transfection with the PRP-4 lentiviral shRNA, puromycin selected clones were plated and exposed to varying concentrations of paclitaxel for 6 days. The cells were fixed and stained with crystal violet to visualize the viable cells, with darker staining representing the more viable cells

Table 2 MISSION™ TRC PRP-4 shRNA Target Set

TRC number	Sequence	Target region	Reverse resistance
TRCN0000000719 (TRC719)	CCGGCCTATCAACTGTCTTATGTACTCGAG TACATAAGACAGTTGATACGGTTTTT	3UTR	Yes
TRCN0000000720 (TRC720)	CCGGCCTGCTGATGTTAAAGAGTATCTCGA GATACTCTTTAACATCAGCAGCTTTTT	CDS	No
TRCN0000000721 (TRC721)	CCGGCTCAAGATCAAGCAAGGAAATCTCGA GATTTCCTTGCTTGATCTTGAGTTTTT	CDS	No
TRCN0000000722 (TRC722)	CCGGGAATGAAAGTTGAGCAGGAATCTCGA GATTCCTGCTCAACTTTCATTCTTTTT	CDS	Yes
TRCN0000000723 (TRC723)	CCGGAGCAAGTCAAAGGAGAGGAAACTCGA GTTTCCTCTCCTTTGACTTGCTTTTTT	CDS	Yes

Confirmation of PRP-4 depletion and effect on paclitaxel sensitivity

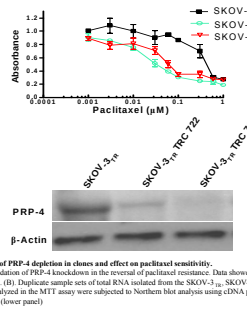

Figure 3. Confirmation of PRP-4 depletion in clones and effect on paclitaxel sensitivity. (A) MTT assay show validation of PRP-4 knockdown in the reversal of paclitaxel resistance. Data showed two of three positive target sites of PRP-4 by shRNA. (B). Duplicate sample sets of total RNA isolated from the SKOV-3$_{TR}$, SKOV-3$_{TR}$/TRC719, and SKOV-3$_{TR}$/TRC720 cell lines analyzed in the MTT assay were subjected to Northern blot analysis using cDNA probe directed against PRP-4 (upper panel) and β-actin (lower panel)

Comparison of PRP-4, MDR1, MDM2, survivin and TOCH1 expression in drug sensitive and resistant cell lines

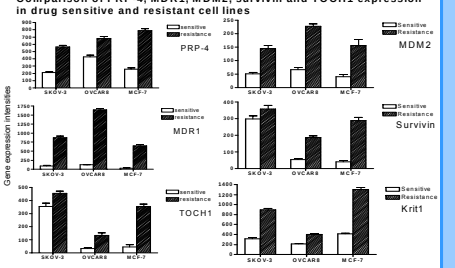

Figure 4. Comparison of PRP-4, MDR1, MDM2, survivin and TOCH1 expression in drug sensitive and resistant cell lines. Affymetrix cDNA array data was extracted, normalized and summarized. The gene expression values and intensities were plotted using GraphPad PRISM® 4 software

Exogenous expression of PRP-4 confers paclitaxel resistance to the ovarian cancer cell line SKOV-3

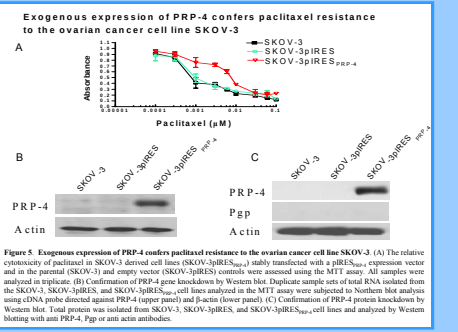

Figure 5. Exogenous expression of PRP-4 confers paclitaxel resistance to the ovarian cancer cell line SKOV-3. (A) The relative cytotoxicity of paclitaxel in SKOV-3 derived cell lines (SKOV-3pIRES$_{neo}$) stably transfected with a pIRES$_{neo}$ expression vector and in the parental (SKOV-3) and empty vector (SKOV-3pIRES) controls were assessed using the MTT assay. All samples were analyzed in triplicate. (B) Confirmation of PRP-4 gene knockdown by Western blot. Duplicate sample sets of total RNA isolated from the SKOV-3, SKOV-3pIRES, and SKOV-3pIRES$_{neo}$ cell lines analyzed in the MTT assay were subjected to Northern blot analysis using cDNA probe directed against PRP-4 (upper panel) and β-actin (lower panel). (C) Confirmation of PRP-4 protein knockdown by Western blot. Total protein was isolated from SKOV-3, SKOV-3pIRES, and SKOV-3pIRES$_{neo}$ cell lines and analyzed by Western blotting with anti PRP-4, Pgp or anti actin antibodies.

CONCLUSIONS

- Inhibition of several target genes by lentiviral shRNA could sensitize SKOV-3$_{TR}$ to paclitaxel .
- Specifically, overexpression of human pre-messenger RNA processing (PRP-4) kinase in ovarian cancer multidrug resistant cells and specific PRP-4 knockdown associated cellular paclitaxel sensitivities .
- Overexpresion of PRP-4 in drug sensitive cells could induce a modest level of drug resistance to paclitaxel.

Acknowledgements: This project was supported by the Ovarian Cancer Research Foundation (OCRF).